Abstract—Salt-sensitive hypertension is associated with impaired NO/cGMP signaling. We hypothesized that increased superoxide production by NADPH oxidase and altered endothelial NO synthase (NOS3) phosphorylation determine endothelial dysfunction in hypertension. Experiments tested if NO/cGMP signaling and NOS3 serine phosphorylation are decreased and NADPH oxidase activity is increased in mesenteric arteries from deoxycorticosterone acetate (DOCA)-salt rats compared with arteries from placebo rats. Concentration response curves to phenylephrine were performed in mesenteric arteries in the presence and absence of N\textsuperscript{G}/H-nitro-L-arginine (LNA) and antioxidants to determine the influence of basal NO and superoxide production on vascular tone. LNA increased phenylephrine sensitivity in arteries from placebo, but not DOCA-salt rats, regardless of antioxidant treatment. To determine basal cGMP production, mesenteric arteries were incubated with 3-isobutyl-1-methylxanthine in the presence or absence of LNA, sodium nitroprusside (SNP), antioxidants, or tetrahydrobiopterin. NOS-dependent cGMP production was reduced in arteries from DOCA-salt rats compared with arteries from placebo rats and was not restored by acute treatment with antioxidants or tetrahydrobiopterin. SNP-induced cGMP production was similar between groups as was NADPH oxidase activity, measured by lucigenin chemiluminescence, in mesenteric arteries. Expression and phosphorylation of NOS3 were examined by Western blotting. Phosphorylation of NOS3 was decreased in arteries from DOCA-salt rats compared with arteries from placebo rats at serine residues 1179 and 635. These findings indicate that diminished NO/cGMP signaling in mesenteric arteries from DOCA-salt rats is caused by reduced phosphorylation of NOS3 at serine 1179 and serine 635, rather than NO scavenging by superoxide. (Hypertension. 2004;43:1080-1085.)

Key Words: mesenteric arteries ▪ cyclic GMP ▪ nitric oxide synthase ▪ phosphorylation ▪ deoxycorticosterone

The endothelium plays a critical role in maintaining vascular tone via the release of vasoactive substances, and endothelial dysfunction is associated with the development of hypertension. While the underlying mechanisms are not completely understood, decreased endothelium-derived nitric oxide (NO) may contribute to endothelial dysfunction. Decreased endothelial NO synthase (NOS3) expression or activity,1 decreased NOS3 substrate or cofactor availability,2–3 or increased degradation of NO by reactive oxygen species (ROS)4 may all contribute to decreased NO bioavailability. Basal production of NO and endothelium-dependent relaxation are attenuated in conduit arteries of deoxycorticosterone acetate (DOCA)-salt hypertensive rats.5–7 However, the role of NO in regulating basal tone in small arteries of DOCA-salt rats has not been resolved.7–9

Oxidative stress contributes to the pathogenesis of hypertension. DOCA-salt hypertension is associated with increased production of superoxide (O\textsuperscript{2–}) via activation of NAD(P)H oxidase in conduit arteries.6,10 O\textsuperscript{2–} reacts rapidly with NO to form peroxynitrite (ONOO\textsuperscript{–}), thereby reducing NO bioavailability. ONOO\textsuperscript{–} and other reactive oxygen species oxidize tetrahydrobiopterin, an essential cofactor of NOS, resulting in decreased NO production.1

Phosphorylation is an important regulator of NOS3 activity. In vitro studies have shown that phosphorylation of NOS3 at serine 1179 (bovine NOS3 sequence, serine 1177 in the human sequence) increases enzyme activity,11–13 and recent evidence suggests that phosphorylation of NOS3 at serine 635 may also positively regulate basal enzyme activity.14–16 Serine residues 116 and 617 have also been identified as phosphorylation sites,13,15 but the functional role of phosphorylation at these sites has not been determined. Because NOS3 phosphorylation may influence vascular tone, it was of interest to investigate the phosphorylation state of NOS3 in small arteries of hypertensive rats.
The purpose of this study was to determine if basal NO/cGMP signaling in small mesenteric arteries of DOCA-salt hypertensive rats is inhibited by NADPH oxidase-dependent \( O_2^- \) production and whether this is accompanied by decreased NOS3 phosphorylation.

**Methods**

**Animals**

DOCA-salt hypertension was created in male Sprague-Dawley rats (200 to 225 g, Harlan Laboratories, Prattville, Ala) as previously described. Two weeks after DOCA-salt treatment, rats were used. Male Sprague-Dawley rats were also performed.

**Isolated Artery Preparation and Vascular Reactivity Protocol**

A third order branch of the superior mesenteric artery was isolated as previously described and placed in the chamber of a wire myograph (Danish Myo Technology A/S) containing physiological saline solution (PSS, mmol/L: 130 NaCl, 4.7 KCl, 1.8 KH\(_2\)PO\(_4\), 1.17 MgSO\(_4\), 7H\(_2\)O, 14.9 NaHCO\(_3\), 5.5 dextrose, 0.26 EDTA, 1.6 CaCl\(_2\)). Cumulative concentration-response curves to phenylephrine (1 to 100 mmol/L) were performed in the presence and absence of the NOS inhibitor N\textsubscript{G}-nitro-L-arginine (LNA, 100 \mu mol/L), the superoxide scavenger tiron (10 mmol/L), or superoxide dismutase-polyethylene glycol (PEG-SOD, 200 U/mL). Cumulative concentration-response curves to KC (8 mmol/L) were also performed.

**Measurement of Intracellular cGMP Content**

The mesenteric arterial bed was isolated as previously described, separated into 4 to 5 sections, and incubated at 37°C in oxygenated PSS with 0.3 mmol/L 3-isobutyl-1-methylxanthine for 10 minutes in the presence or absence of 100 \mu mol/L LNA, 1 \mu mol/L sodium nitroprusside (SNP), 300 U/mL SOD, 200 U/mL PEG-SOD, or 10 \mu mol/L tempol. Preincubation was performed with either PEG-SOD or tempol for 5 minutes prior to the addition of 1 mmol/L tetrahydrobipterin for 5 minutes. Maximal force generation in response to phenylephrine was determined by standard Bradford assay (Bio-Rad Laboratories) using bovine serum albumin as the standard.

**Immunoblotting**

The entire mesenteric arterial bed was homogenized, and Western blotting was performed as previously described. Primary antibodies included mouse monoclonal anti-NOS3 (Transduction Laboratories); rabbit polyclonal antibodies for phosphorylated NOS3-Ser\textsuperscript{635}, Akt-Ser\textsuperscript{473}, Akt-Thr\textsuperscript{308}, and total Akt (Cell Signaling); and rabbit polyclonal antibody for phosphorylated NOS3-Ser\textsuperscript{116} (Upstate Biotechnology). Rabbit polyclonal antibodies for phosphorylated NOS3-Ser\textsuperscript{617} and NOS3-Ser\textsuperscript{617} were raised and purified as previously described. Equal protein loading was verified by \( \beta\)-actin immunoblotting (Sigma).

**NADPH Oxidase Assay**

\( O_2^- \) detection experiments were conducted in 96-well microplates (OptiPlate-96, Perkin-Elmer) to 25 \mu g of mesenteric artery homogenate or PSS was added to sample and background wells, respectively, and incubated for 30 minutes at 37°C. Enzymatic activity was measured by lucigenin chemiluminescence (5 \mu mol/L in the presence of 100 \mu mol/L NADPH (Alexis Biochemicals). After a 30-minute dark-adapt period, plates were counted on a TopCount Microplate Scintillation and Luminescence Counter (Perkin Elmer) set to single-photon counting mode. Enzyme activity was expressed as cpm/\mu g protein. No O\textsubscript{2} production was detected in the absence of NADPH, and \( O_2^- \) detection was diminished by tempol (10 mmol/L).

**Data Analysis**

Values are expressed as mean ± SEM. Phenylephrine and KC concentration-response curves were expressed as percent of maximum constriction and analyzed using nonlinear regression of sigmoidal dose-response curves (GraphPad Prism; San Diego, Calif), which were used to calculate the EC\textsubscript{50}. Maximum response and log EC\textsubscript{50} values were compared using 1-way ANOVA, and individual comparisons were performed using a Student-Newman-Keuls test. DOCA-salt/placebo comparisons were performed using a t test for independent samples. A probability value <0.05 was considered significant.

**Results**

Two-week DOCA-salt treatment significantly increased systolic blood pressure compared with placebo (197±2 mm Hg and 135±4 mm Hg, respectively, \( n = 9 \) to 10, \( P < 0.0001 \)).

**Vasoconstrictor Responses**

To determine the influence of NOS and \( O_2^- \) on basal tone of mesenteric arteries of DOCA-salt and placebo rats, responses to phenylephrine were measured in the presence and absence of LNA, tiron, or PEG-SOD. Incubation with LNA significantly increased phenylephrine sensitivity in arteries from placebo rats with or without antioxidant treatment but did not alter responses to phenylephrine in untreated arteries from DOCA-salt rats (Figure 1 and Table, \( n = 8 \) to 15). Mesenteric arteries from DOCA-salt rats were significantly less sensitive to phenylephrine compared with arteries from placebo rats (Figure 1, Table, log EC\textsubscript{50} = 5.8±0.7 mmol/L and 6.4±0.1 mmol/L, respectively, \( n = 15 \) to 18, \( P < 0.05 \)). However, there was no difference in the sensitivity to phenylephrine between arteries from DOCA-salt rats treated with the antioxidants tiron or PEG-SOD and antioxidant-treated arteries from placebo rats (Table, \( n = 7 \) to 11). Combined antioxidant and LNA treatment increased phenylephrine sensitivity compared with untreated arteries (Figure 2 and Table, \( n = 7 \) to 15). Maximal force generation in response to phenylephrine was not altered by DOCA-salt, LNA, or antioxidant treatments (\( n = 7 \) to 18). Sensitivity and maximal force generation in response to KC were similar in arteries from DOCA-salt and placebo rats (Table, \( n = 7 \) to 8).

**cGMP Content**

Basal NO production in mesenteric arteries of DOCA-salt and placebo rats was indirectly measured by determining cGMP levels in mesenteric arteries. As shown in Figure 3, cGMP levels were significantly greater in mesenteric arteries from placebo rats compared with arteries from DOCA rats (175±26 and 101±23 pmol/mg protein, respectively, \( P < 0.05, n = 14 \)). LNA significantly reduced cGMP levels in arteries from DOCA-salt rats (Figure 1 and Table, log EC\textsubscript{50} = 5.8±0.7 mmol/L and 6.4±0.1 mmol/L, respectively, \( n = 15 \) to 18, \( P < 0.05 \)). However, there was no difference in the sensitivity to phenylephrine between arteries from DOCA-salt rats treated with the antioxidants tiron or PEG-SOD and antioxidant-treated arteries from placebo rats (Table, \( n = 7 \) to 11). Combined antioxidant and LNA treatment increased phenylephrine sensitivity compared with untreated arteries (Figure 2 and Table, \( n = 7 \) to 15). Maximal force generation in response to phenylephrine was not altered by DOCA-salt, LNA, or antioxidant treatments (\( n = 7 \) to 18). Sensitivity and maximal force generation in response to KC were similar in arteries from DOCA-salt and placebo rats (Table, \( n = 7 \) to 8).
Figure 1. Cumulative concentration response curves to phenylephrine (PE) in mesenteric arteries of placebo (●) and DOCA-salt (▲) rats in the presence (unfilled symbols) and absence (filled symbols) of LNA (100 μmol/L). Data are plotted as percentage of maximum contractile response to PE. n=7 to 18. *Significant shift in EC50 vs no LNA (P<0.05).

basal), or tempol (72±22% of basal, n=6 to 9). Addition of tetrahydrobiopterin after pretreatment of arteries with PEG-SOD (129±32% of PEG-SOD alone, n=4) or tempol (76.1±18% of tempol alone, n=10) did not affect cGMP levels in arteries from DOCA-salt rats.

Figure 2. Shift in phenylephrine sensitivity compared with DOCA control in response to LNA (100 μmol/L) and antioxidants (tiron, 10 mmol/L and PEG-SOD, 200 U/mL). n=7 to 8. *Significant shift in EC50 vs untreated DOCA (P<0.05).

NADPH Oxidase Activity
We examined NADPH oxidase-dependent superoxide production in the mesenteric arterial bed because acute antioxidant treatment did not affect NO/cGMP signaling. NADPH oxidase activity in mesenteric arteries was similar between groups (placebo: 4228±216 cpm/μg protein, DOCA: 4266±640 cpm/μg protein, n=7). Superoxide detection in the presence of NADPH was suppressed by tempol (placebo: 266±17 cpm/μg protein, DOCA: 294±34 cpm/μg protein, n=7), thus verifying the specificity of the assay.

Expression and Phosphorylation of NOS3 and Akt in Mesenteric Arteries
Because serine phosphorylation of NOS3 regulates enzyme activity, we examined the expression and phosphorylation of NOS3 in mesenteric arteries of DOCA-salt and placebo rats. Total NOS3 expression was similar between groups (placebo: 4228±75 densitometric units [DU], DOCA: 719±135 DU, n=6). The ratio of phosphorylated NOS3:total NOS3 of serine 1179 (Figure 5, 0.4±0.1 and 1.1±0.2, respectively, P<0.01, n=6) and serine 635 (0.5±0.2 and 1.1±0.2, respectively, P<0.05, n=6). The ratio of phosphorylated NOS3:total NOS3 was not different at serines 116 (placebo=2.8±1.1, DOCA=2.1±1.0, n=5) or 617 (placebo=1.0±0.2, DOCA=0.7±0.2, n=5). As shown

Sensitivity and Maximum Force Generation in Response to Phenylephrine and KC in of Isolated Mesenteric Arteries From Placebo and DOCA-Salt Rats

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Placebo</th>
<th>DOCA-Salt</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>log EC50 (mol/L)</td>
<td>Maximum Response (mN)</td>
</tr>
<tr>
<td>PE Control</td>
<td>-6.4±0.1</td>
<td>15.8±0.6</td>
</tr>
<tr>
<td>PE + LNA</td>
<td>-6.8±0.2*</td>
<td>14.4±0.9</td>
</tr>
<tr>
<td>PE + Tiron</td>
<td>-6.4±0.1</td>
<td>16.8±0.8</td>
</tr>
<tr>
<td>PE + Tiron + LNA</td>
<td>-6.8±0.1†</td>
<td>19.0±1.3</td>
</tr>
<tr>
<td>PE + PEG-SOD</td>
<td>-6.3±0.1</td>
<td>15.3±1.3</td>
</tr>
<tr>
<td>PE + PEG-SOD + LNA</td>
<td>-6.6±0.1‡</td>
<td>17.2±1.0</td>
</tr>
<tr>
<td>KCl</td>
<td>-1.4±0.2</td>
<td>10.3±0.4</td>
</tr>
</tbody>
</table>

Data obtained from experiments described in Figure 1. All abbreviations are defined in the text.

*P<0.05 vs placebo control; †P<0.05 vs tiron alone; ‡P<0.05 vs PEG-SOD alone; §P<0.05 vs DOCA-salt control.
in Figure 6, the ratio of phosphorylated Akt:total Akt DU at both threonine 308 (placebo/H11005 0.8/H11006 0.2, DOCA/H11005 1.1/H11006 0.1, n=5) and serine 473 (placebo/H11005 0.6/H11006 0.1, DOCA/H11005 0.6/H11006 0.1, n=5) was similar between groups. No change in total Akt protein expression was observed (placebo/H11005 2817/H11006 275 DU, DOCA/H11005 3283/H11006 315 DU, n=5).

**Discussion**

The purpose of this study was to assess basal NO/cGMP signaling in small mesenteric arteries of DOCA-salt and placebo rats and elucidate mechanisms that may determine NO bioavailability. We found that basal NO/cGMP signaling is diminished in small mesenteric arteries of DOCA-salt rats, and this decrease is associated with reduced NOS3 phosphorylation at two important positive regulatory sites: serine 1179 and serine 635.

To assess the contribution of basal NO production in the maintenance of basal tone of small mesenteric arteries of DOCA-salt and placebo rats, contractile responses of arteries to phenylephrine were measured in the absence and presence of LNA. Contraction to phenylephrine was reduced in mesenteric arteries after 2-week DOCA-salt treatment, suggesting that increased blood pressure observed in early stages of DOCA-salt hypertension is not due to enhanced sensitivity to adrenergic stimuli. This is consistent with previous studies that demonstrate contractile sensitivity to agonists such as norepinephrine, phenylephrine, or endothelin is decreased in resistance arteries after 2 to 3 weeks of DOCA-salt hypertension.7,8,23 However, other studies have shown that the sensitivity of mesenteric arteries to phenylephrine is unchanged24 or increased25,26 after 4 to 9 weeks of DOCA-salt hypertension. This discrepancy may be due to the different methods used to assess vascular reactivity or differences in the duration of hypertension. Interestingly, phenylephrine sensitivity in arteries from DOCA-salt rats was significantly increased following combined antioxidant and LNA treatment, suggesting that reduced sensitivity to phenylephrine in arteries from DOCA-salt rats may be mediated by a vasodilatory ROS, possibly produced by uncoupled NOS. Antioxidant treatment had no effect on phenylephrine responses in arteries from placebo rats.

NOS inhibition significantly increased sensitivity to phenylephrine in arteries from placebo rats; however, NOS inhibition did not alter phenylephrine responses in arteries from DOCA-salt animals, suggesting a loss of NOS-dependent antagonism to contraction in DOCA-salt hypertension. This is supported by the finding that NOS-dependent cGMP production is reduced in mesenteric arteries of DOCA-salt rats. Placebo and DOCA-salt treated rats had similar cGMP production in response to the NO donor SNP, indicating no dysfunction in the response to exogenous NO. Consistent with the observed basal contractile responses to phenylephrine, these results indicate that there is a loss of basal NO bioavailability in mesenteric arteries from DOCA-salt rats. This loss of NO/cGMP signaling could contribute to increased peripheral vascular resistance in this model of hypertension.

Increased production of ROS can contribute to endothelial dysfunction by reducing NO bioavailability. Hypertension is associated with oxidative stress; however, $O_2^-$ production...
and NADPH oxidase activity are increased in conduit arteries of DOCA-salt rats, thus the observed decrease in NO/cGMP signaling does not appear to be caused by accelerated degradation of NO due to increased \( \cdot O_2^- \) production.

ROS such as \( \cdot O_2^- \) and ONOO\(^-\) oxidize the NOS cofactor tetrahydrobiopterin, reducing NO production.\(^{27}\) Studies have shown that there is increased oxidation of tetrahydrobiopterin in the aorta of DOCA-salt hypertensive mice and that oral tetrahydrobiopterin treatment of DOCA-salt mice prevented increases in blood pressure and NO uncoupling in the aorta.\(^3\) It has also been shown that 1-hour treatment of aortas from apoE\(^/-\) mice with sepiapterin, a precursor to tetrahydrobiopterin, improved endothelial function.\(^{27}\) In contrast, we found that acute treatment of mesenteric arteries with tetrahydrobiopterin, in the presence of antioxidants, had no effect on cGMP levels, suggesting that oxidation of tetrahydrobiopterin does not contribute to the observed decline in NO/cGMP signaling. This discrepancy may reflect differences in levels of oxidant stress between conduit arteries and small arteries.

We next examined whether the observed loss of NO-dependence in basal tone and decrease in NO/cGMP signaling may be due to changes in NOS3 expression or phosphorylation. Phosphorylation of NOS3 at serine 1179 increases catalytic activity by reducing \( Ca^{2+} \) dependence,\(^{12,13}\) and phosphorylation at serine 635 increases NO production.\(^{14–16}\) Although total expression of NOS3 was similar in mesenteric arteries from placebo and DOCA-salt rats, phosphorylation of NOS3 at both serine 1179 and serine 635 was decreased in mesenteric arteries of DOCA-salt hypertensive rats compared with arteries from placebo rats. NOS3 phosphorylation at serine 116 and serine 617 was similar between groups. This is consistent with the NOS phosphorylation pattern being responsible for the decreased NO/cGMP signaling in the mesenteric arteries of these hypertensive rats because mutation studies\(^{16}\) indicate that serine 617 negatively regulates basal and stimulated NO release and serine 116 only contributes to agonist-stimulated NO release. The specific decrease in basal phosphorylation of NOS3 at serine residues 1179 and 635 is expected to diminish enzyme activity and NO production, therefore contributing to decreased NO/cGMP signaling in mesenteric arteries of DOCA-salt rats.

Previous studies have shown that NOS3 is directly phosphorylated and activated by the serine/threonine kinase Akt.\(^{12}\) In our studies, expression and phosphorylation of Akt were similar in arteries from placebo and DOCA-salt rats, indicating that changes in activation of Akt by phosphorylation may not be responsible for the observed decrease in phosphorylation of NOS3. These observations were made in homogenates of the entire mesenteric arterial bed, so the presence of Akt in vascular smooth muscle may mask changes in the expression or phosphorylation of Akt in endothelial cells; however, Akt is not the only kinase responsible for phosphorylation of NOS3.\(^{28}\) NOS3 can also be phosphorylated at both serine 1179 and serine 635 by cAMP- and cGMP-dependent protein kinases.\(^{14,28,29}\) NOS3 activity may also be affected by phosphatases, including both PP1 and PP2A.\(^{30,31}\) Changes in the activities of these kinases or phosphatases may be responsible for the observed decrease in NOS phosphorylation, but these pathways have yet to be explored in vivo.

**Perspectives**

There is considerable in vitro evidence that NOS3 phosphorylation is an important regulator of enzyme activity. Our present findings provide novel insights into the role of phosphorylation in NOS3 regulation in vivo. Mesenteric arteries from hypertensive rats have impaired NO/cGMP signaling that may be attributed to reduced NOS3 phosphorylation at serine residues 1179 and 635. In small resistance arteries of hypertensive animals, changes in kinase and phosphatase activity may result in dysregulation of NOS3 and decreased NO bioavailability that is independent of NADPH oxidase-dependent \( O_2^- \) production.
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Jennifer M. Sasser, Jennifer C. Sullivan, Ahmed A. Elmarakby, Bruce E. Kemp, David M. Pollock and Jennifer S. Pollock

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